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We have begun to analyze human T cell responses to dengue antigens in vitro to elucidate the possible role of T lymphocytes in the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome. Dengue antigens induce proliferative responses of peripheral blood mononuclear cells (PBMC) from dengue antibody-positive donors, but do not induce specific proliferative responses of PBMC from dengue antibody-negative donors. Interferon gamma (IFN) is detected in the culture fluids of dengue-immune PBMC stimulated with dengue antigens. Dengue-specific T cell clones were established using lymphocytes from a donor who was known to be infected with dengue 3 virus. These clones respond best to dengue 3 Ag, but they also respond to dengue 1, 2 and 4 antigens to some levels. They have CD3+, CD4+ and CD8- phenotypes, and produce IFN in response to dengue Ag. One clone examined to date has cytotoxic activity to dengue-infected autologous cells.

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IFN (gamma)

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The effect of IFN γ on dengue virus infection of human monocytic cells was studied. Pretreatment of U937 cells with IFN-γ results in a significant increase in the number of dengue-infected cells and in the yield of infectious virus. IFN-γ does not augment dengue virus infection when cells are infected with virus in the absence of anti-dengue antibodies. IFN γ does not augment dengue virus infections when cells are infected with virus in the presence of $F(ab')_2$ prepared from anti-dengue IgG. Human γ- globulin inhibits IFN-γ induced augmentation. IFN-γ increases the number of Fc γ receptors on U937 cells. The increase in the percentage of dengue antigen-positive cells correlate with the increase in the number of Fc γ receptors after rIFN-γ treatment. These results indicate that IFN-γ-induced augmentation of dengue virus infection is Fc γ receptor-mediated. IFN γ augments dengue virus infection of human monocytes enriched from PBMC.

These result indicate that PBMC from dengue-immune donors contain CD4(+) T cells which proliferate and produce IFN γ after stimulation with dengue antigens, and suggest that the IFN γ which is produced by these stimulated dengue-specific T cells may contribute to the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome by increasing the number of dengue virus-infected monocytes in the presence of cross-reactive anti-dengue antibodies. (AUS)

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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

ANNUAL REPORT

FRANCIS A. ENNIS

JULY 31, 1988

Supported by

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FOREWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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I. INTRODUCTION

Dengue infections are a major cause of morbidity worldwide, and hemorrhagic fever and shock are very severe and frequently fatal complications of dengue infections (1). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a different dengue serotype than they experienced as their primary infection (2). It has been speculated that dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are mediated by host immune mechanisms.

We have begun to define the role of dengue-specific T lymphocytes in the pathogenesis of DHF/DSS. To address this question we established dengue-specific T cell clones. In this report we describe the characteristics of these clones. They have $CD3^+$, $CD4^+$, $CD8^-$ phenotypes and respond to four serotypes of dengue antigens with the highest response to dengue 3 antigen. They produce IFNγ after stimulation with dengue antigens. The IFNγ produced augments dengue virus infection of human monocytes and monocytic cells in the presence of anti-dengue antibody.

II. RESULTS

A. Dengue virus-specific T cell responses in bulk culture

A-1. Proliferative responses of PBMC from dengue-immune donors induced by dengue antigens

We attempted to induce dengue antigen-specific proliferative responses of lymphocytes. Sonicated, dengue-infected Vero cells were used as dengue antigens. PBMC from a dengue antibody-positive donor were cultured with dengue or control antigens diluted at various concentrations, and ^{3}H-TdR incorporation was examined. Dengue antigens induced significant proliferative responses of PBMC, and there was a good correlation between the level of the proliferative responses and the concentration of dengue antigens (Figure 1). Control antigen did not induce significant proliferative responses. A time course study showed that the proliferative responses reached maximum levels on day 6 (data not presented).

The results shown in Figure 2 illustrate the proliferative responses of PBMC obtained from eleven Thai donors and two American donors who were known to have previously been infected with dengue virus. The PBMC from these donors showed significant proliferative responses induced by dengue antigens (8/9 with dengue 1 Ag; 10/13 with dengue 2 Ag; 5/7 with dengue 3 Ag; 5/7 with dengue 4 Ag). The PBMC from Massachusetts blood bank donors did not significantly proliferate in response to dengue antigens. The failure of PBMC of some Thai donors to respond to certain dengue antigens may be due in part to the fact that PBMC were derived from healthy adults, and dengue infections are most common during childhood. Table 1 contains a summary of the positive proliferative responses induced by dengue antigens using PBMC from Thai and American donors. These results indicate that antigens of the four dengue serotypes can induce proliferative responses using PBMC from dengue antibody-positive donors. Neither dengue antigens nor control antigens induced significant proliferative responses of PBMC from non-immune donors.

Figure 1: Proliferative responses of dengue-immune PBMC to dengue Ag. 2 x 10^5 PBMC from an American donor who had been infected with dengue 3 virus were cultured with dengue 3 Ag or control Vero Ag at various dilutions for 6 days. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before being harvested. ○: Proliferative responses induced by dengue 3 antigen. ●: Proliferative responses induced by control antigen.

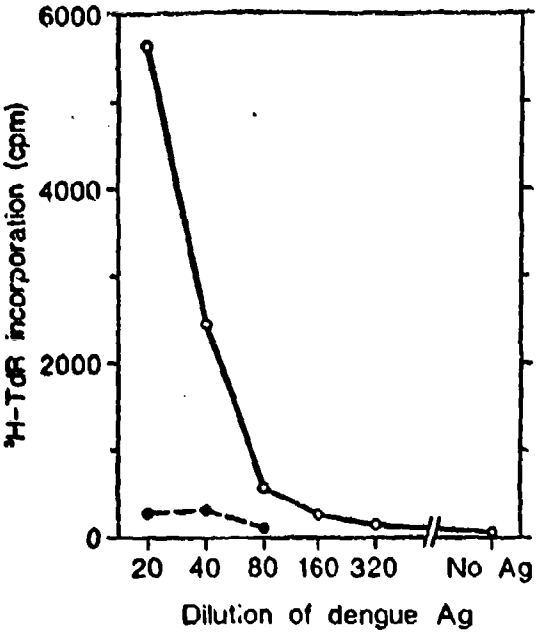

Figure 2: Proliferative responses of PBMC from eleven Thai and two American antibody-positive donors. 4 x 10^5 PBMC were cultured with dengue and control Vero antigens duluted at 1:30 for 6 days. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest. Results were expressed as stimulation index which was calculated as follows: mean CPM induced by dengue Ag/mean CPM induced by control Ag. Stimulation indices greater than 2 were considered as positive proliferative responses. D: Dengue-immune donors. N: Non-immune donors.

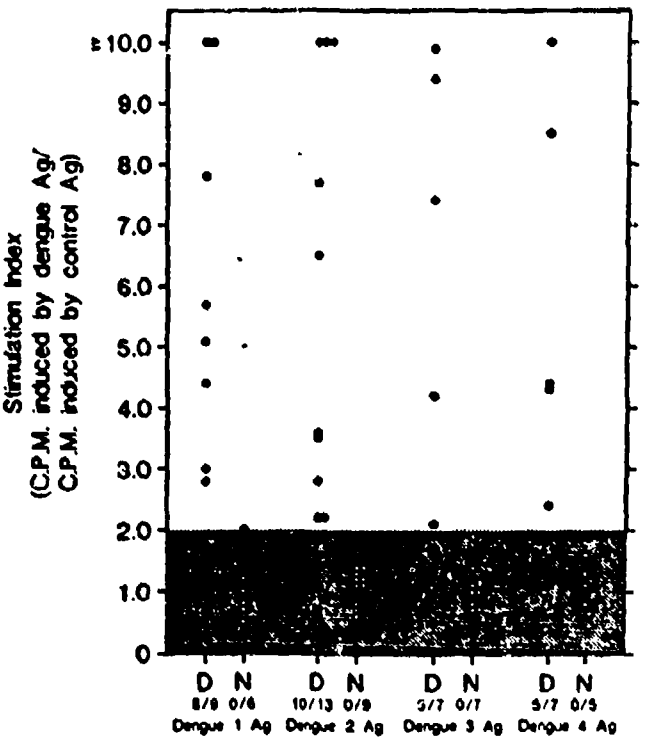

Table 1: A summary of the proliferative responses of PBMC from dengue immune donors to dengue antigens.[a]

	Dengue-immune[b]			Non-immune		
		^{3}H-TdR incorporation			^{3}H-TdR incorporation	
	No. of donors	Stimulation Index	c.p.m.	No. of donors	Stimulation Index	c.p.m.
Dengue 1	8	7.4 (2.8-15.7)	9011 (1018-19343)	6	1.0 (0.5-2.0)	664 (142-2357)
Dengue 2	10	7.5 (2.2-19.5)	4644 (1019-10872)	9	1.1 (0.6-1.4)	1038 (93-2897)
Dengue 3	5	6.6 (2.1-9.9)	6005 (1706-13762)	7	1.1 (0.7-1.9)	732 (158-1975)
Dengue 4	5	8.2 (2.4-21.5)	7936 (2153-17178)	5	1.3 (0.8-1.8)	611 (185-1275)
Control Ag	11	-	1046 (68-4033)	9	-	783 (261-2172)
No Ag	11	-	630 (69-1980)	9	-	529 (95-1723)

[a] 4×10^5 PBMC were cultured for 6 days with dengue or control Ag diluted at 1:30. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest. Results are presented as averages.

[b] The responses of PBMC of dengue-immune donors who had a stimulation index of greater than two with each of the four dengue antigens are included. The stimulation index was calculated from mean CPM induced by dengue Ag/mean CPM induced by control Ag. Quadruplicate samples were used.

A-2. Detection of IFNγ in the culture fluids of PBMC stimulated with dengue antigens

We examined the culture fluids of PBMC stimulated with dengue antigens for IFN using ELISA with monoclonal antibodies to human IFNγ and α. IFNγ was detected at high titer in the culture fluids of PBMC from dengue-immune donors stimulated with dengue antigens ($p<0.02$ compared to the amount of IFNγ detected in cultures of PBMC from non-immune donors), but not in the culture fluids of PBMC stimulated with control antigen or cultured alone (Table 2). IFNα was not detected in the culture fluid of PBMC after stimulation with dengue or control antigen (data not presented). The culture fluids of PBMC from antibody-negative donors stimulated with dengue antigens did not contain significant titers of IFNs. These results indicate that dengue-immune PBMC produce high titers of IFNγ after stimulation with dengue antigens, but do not produce IFNα.

IFNγ was detected at low titers in the culture fluids of PBMC from some donors cultured without antigens. It has been reported that PBMC from some normal adults produce IFNγ during in vitro culture without the addition of specific antigens.

Table 2: IFNγ production by PBMC from dengue antibody-positive donors after stimulation with dengue antigens.[a]

	IFNγ (U/ml)[b]					
Donors	Dengue 1 Ag	Dengue 2 Ag	Dengue 3 Ag	Dengue 4 Ag	Control Ag	No Ag
Dengue antibody-positive						
T1	25	120	28	70	2	2
T2	42	56	51	115	14	14
T3	33	47	14	26	1	3
A1	32	43	61	38	3	3
Dengue antibody-negative						
M1	8	5	8	5	5	6
M2	3	11	4	3	2	3
M3	<1	2	2	2	2	2
M4	<1	<1	<1	<1	<1	<1

[a] 4×10^5 PBMC were cultured for 6 days with dengue and control Ag diluted at 1:30. Culture fluids were examined for IFNγ by ELISA.

[b] The titers of IFNγ induced by dengue and control antigens were compared by Student's t test between dengue antibody-positive and antibody-negative donors. IFNγ induced by dengue 1Ag, $p<0.001$; by dengue 2 Ag, $p<0.02$; by dengue 3 Ag, $p<0.02$; by dengue 4 Ag, $p<0.01$; by control Ag, $p>0.2$ (not significant); without Ag, $p>0.2$ (not significant).

B. Human T cell responses to dengue viruses at a clonal level

B-1. Establishment of dengue-specific T cell clones using a limiting dilution method

We used PBMC from donor A who was known to be infected with dengue 3 virus for cloning. Table 3 shows proliferation responses of donor A PBMC to dengue antigens in a bulk culture.

Table 3: Proliferative responses of the PBMC of donor A to dengue antigens in a bulk culture

Antigens	^{3}H-TdR incorporation (c.p.m.)		
	Donor A	Donor B*	Donor C**
Dengue 1	5128	15932	481
Dengue 2	6643	5983	516
Dengue 3	25177	6772	682
Dengue 4	2883	4329	603
Control	660	1065	460
No Ag	707	1091	450

*Donor B was known to have been infected with dengue 1 virus in Aruba in 1985.
**Donor C did not have anti-dengue antibody.

4 x 10^5 PBMC were cultured with dengue 3 antigen at a final concentration of 1:30 in 0.2 ml RPMI containing 10% human AB serum in 96 well round-bottom plates for 7 days. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation and were cultured at concentrations of 1 cell/well with γ-irradiated autologous PBMC (1 x 10^5) in 0.2 ml RPMI containing 10% human AB serum (UMMC), 10% IL-2 and dengue 3 antigen at a final dilution of 1:30 in 96 well round bottom plates. On day 14, 0.1 ml of medium was removed from each well and 0.1 ml of fresh medium with human AB serum, IL-2 and dengue antigen was added to maintain the same final concentrations described above. On day 21, cells in wells demonstrating growth were transferred to 48 well flat bottom plates (Costar, Cambridge, MA) and were further cultured with 1 x 10^6 γ-irradiated autologous PBMC in 1 ml of RPMI containing 10% human AB serum, 10% IL2, and dengue antigen at a dilution of 1:30.

B-2. Antigen specificities of the clones

Fifteen clones were established and were examined for antigen specificity using dengue antigens of four serotypes. All the clones responded best to dengue 3 antigen, and they also responded to dengue 1, 2 and 4 antigens to lower but significant levels (Table 4). Some clones also responded to yellow fever antigen. Therefore, they are dengue virus-specific and serotype cross reactive.

Table 4: Proliferative responses of human T cell clones to dengue antigens

Clones	^{3}H-TdR incorporation (c.p.m.)						
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	YF	Control Ag	No Ag
JK3b	2322	1960	4816	1968	1226	398	200
JK3c	2052	1760	5482	1120	624	596	500
JK3e	1546	1630	2906	1238	456	620	356
JK24	2581	2205	13809	1457	355	348	108
JK26	1438	2078	2375	1394	641	436	708
JK27	6683	5711	11728	3839	1880	827	650
JK28	1949	2286	2600	1681	788	684	633
JK30	831	896	3246	721	276	112	218
JK31	745	1269	7309	837	397	140	133
JK32	1416	3290	4102	2410	1925	133	245
JK33	4530	8152	12756	6286	3070	1161	1949
JK34	2651	8457	9256	4253	1910	480	1076
JK35	3507	4372	6260	5025	3854	2403	1290
JK36	1732	13159	15055	7167	903	140	1225
JK37	4478	6958	26892	5827	2546	1223	1985

B-3. Phenotypic analysis of the clones

Phenotypes of the clones were examined using five monoclonal antibodies. Table 5 indicates specificities of the monoclonal antibodies used.

Table 5: Specificities of the monoclonal antibodies against human T cells

CD3 (anti-Leu4)	panT
CD4 (anti-Leu3)	helper/inducer
2H4	suppressor inducer
4B4	helper for Ab production
CD8 (anti-Leu2)	suppressor/cytotoxic

All the clones had $CD3^+$, $CD4^+$ and $CD8^-$ phenotypes. Six clones examined (JK26, 31, 32, 34, 35, 37) were $2H4^-$ $4B4^+$, and one clone (JK33) was $2H4^-$ $4B4^-$.

B-4. Lymphokine production by dengue-specific clones

The clones were examined for the production of IFNγ and lymphotoxin (LT) after stimulation with dengue and yellow fever antigens. They produced high titers of IFNγ after stimulation of dengue 3 Ag. They also produced IFNγ after stimulation with dengue 1, 2 and 4 antigens. The titers of IFNγ induced by dengue 1, 2 and 4 antigens were not as high as those induced by dengue 3

antigen (Table 6). They did not produce LT by the stimulation with specific dengue antigens.

Table 6: IFNγ production by T cell clones after stimulation with antigens

	IFNγ (U/ml)						
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	YF	Control Ag	No Ag
JK3b	2	3	11	7	6	7	4
JK3c	4	3	16	2	5	6	7
JK3e	3	6	10	4	2	5	3
JK24	33	4	53	5	3	<1	<1
JK26	26	74	39	19	9	6	7
JK27	7	6	35	4	3	3	3
JK28	13	14	13	6	2	1	<1
JK30	1	3	15	3	<1	3	<1
JK31	3	3	8	2	2	5	6
JK32	12	23	18	11	6	3	3
JK33	14	24	41	17	8	2	5
JK34	6	22	23	10	4	2	3
JK35	17	33	51	21	10	<1	3
JK36	2	15	18	7	2	3	<1
JK37	3	7	53	23	4	4	4

B-5. Lysis of dengue virus-infected autologous target cells by JK32

JK32 clone was examined for cytotoxic activity to dengue virus-infected cells. We used as target cells dengue 2 virus-infected, Epstein-Barr Virus (EBV)-transformed autologous lymphoblastoid cells, uninfected EBV-transformed autologous lymphoblastoid cells and K562 cells. JK32 lysed dengue-infected autologous target cells, but did not lyse uninfected target cells or K462 cells (Table 7). Time course study showed that significant lysis was detected at 2 hours of incubation and the lysis reached maximum levels at 6 hours (Table 8).

Table 7: Lysis of dengue 2-infected autologous cells by JK32

Effector/Target ratio	% specific ^{51}Cr release[a]		
	Dengue-infected autologous cells	Uninfected autologous cells	K562
10	60	5	1
3	57	1	1
1	49	0	0
0.3	28	0	0

[a] 5×10^3 target cells were incubated with various numbers of effector cells for 6 hours.

Table 8: Time course of lysis of dengue 2-infected autologous cells by JK32

Incubation time (hours)	% specific ^{51}Cr release[a]	
	Dengue-infected target cells	Uninfected target cells
1	6	0
2	23	1
3	40	2
4	59	2
6	83	10
10	87	9

[a]Effector/target ratio was 2.

We then examined the effects of monoclonal antibodies on the lysis of dengue-infected target cells by JK32. OKT3 and OKIa1 antibodies inhibited the lysis, but OKT1, W6.32 or BBM.1 did not (Table 9). These results indicate that JK32 lyse target cells via the CD3 molecule and in a HLA-DR restricted fashion.

Table 9: OKT3 and OKIa1 inhibit lysis of dengue-infected target cells by JK32

	Monoclonal antibodies	% inhibition
Exp. 1	OKT1	0
	OKT3	70
	OKIa1	72
	W6.32	22
Exp. 2	OKT3	71
	BBM.1	17

C. IFNγ augments dengue virus infection of human monocytic cells in the presence of anti-dengue antibody

We examined the effect of IFNγ on dengue virus infection of human monocytic cells.

C-1. IFNγ augments dengue virus infection of U937 cells in the presence of anti-dengue antibody

U937 cells were incubated with 100 U/ml of recombinant IFN-γ (rIFN-γ) for 24 hours, and then were infected with dengue virus at a m.o.i. of 5 p.f.u./cell

in the presence of anti-dengue mouse serum. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescence staining 24 hours after infection. Anti-dengue serum at final dilutions of $1:10^3$, 10^4, and 10^5 augmented infection of nontreated U937 cells, and further augmented dengue virus infection, when U937 cells were pretreated with rIFN-γ (Figure 3). Normal mouse serum which did not contain detectable levels of anti-dengue antibody did not augment dengue virus infection of the nontreated or the IFN-γ treated U937 cells (data not presented). Based on these results, we decided to use anti-dengue serum at a final dilution of $1:10^4$ in the following experiments.

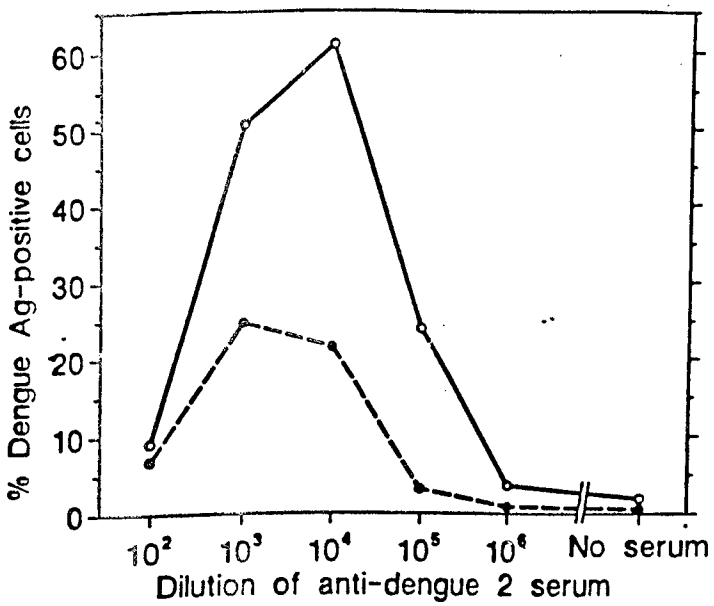

Figure 3: Effect of dilution of anti-dengue serum on dengue virus infection of U937 cells. U937 cells were incubated with or without 100 Uml of rIFN-γ for 24 hours, and infected with dengue virus at a m.o.i. of 5 p.f.u./ml in the presence of variable dilutions of anti-dengue mouse serum. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescence staining 24 hours after infection. ○: U937 cells pretreated with rIFN-γ.
●: Nontreated U937 cells.

U937 cells were incubated with variable amounts of rIFN-γ for 24 hours, and infected with dengue virus at a m.o.i. of 5 p.f.u./cell in the presence of the anti-dengue mouse serum at a final dilution of $1:10^4$. Pretreatment of U937 cells with rIFN-γ at concentrations from 1 to 10,000 U/ml increased the percentage of dengue antigen-positive cells. The percentage of antigen-positive cells reached a maximum level by pretreatment with IFN-γ at 100 U/ml (Figure 4). When U937 cells were infected with dengue virus in the absence of anti-dengue antibody, pretreatment with rIFN-γ did not increase the percentage of antigen-positive cells (1-3% without rIFNγ-treatment and 1-4% with rIFNγ-treatment). Pretreatment of U937 cells with 1-1000 U/ml of rIFN-γ also increased dengue virus titers detected in the culture fluids when cells were infected with virus in the presence of antibody, but not when cells were infected in the absence of antibody (Table 10).

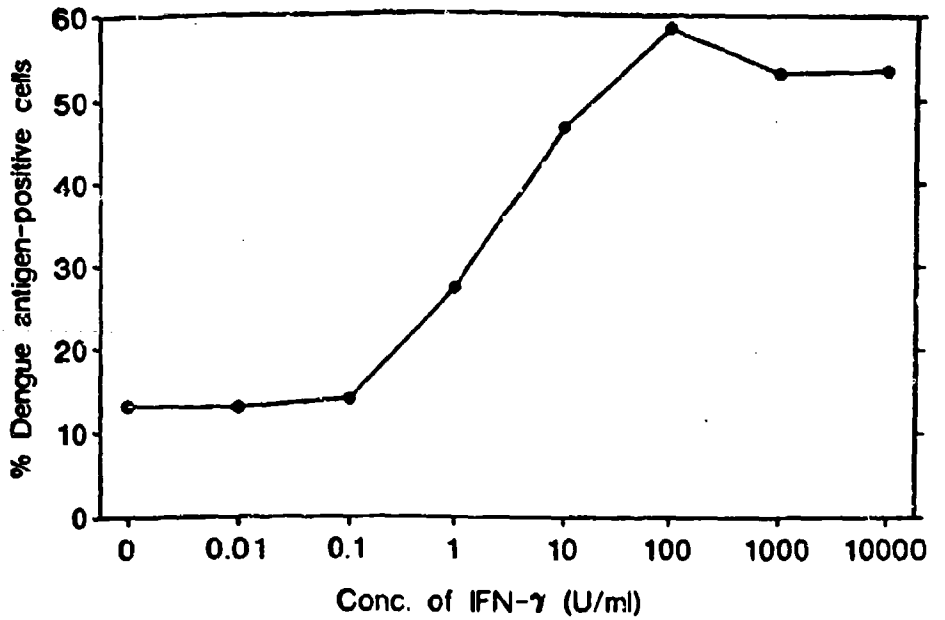

Figure 4: Dengue virus infection of U937 cells pretreated with IFN-γ. U937 cells were incubated with variable concentrations of IFN-γ for 24 hours and then infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescent staining 24 hours after infection.

Table 10: Virus titers in the culture fluids of dengue-infected U937 cells pretreated with IFN-γ.[a]

IFN-γ (U/ml)	Virus titer (p.f.u./ml)	
	Infection in the presence of anti-dengue antibody	Infection in the absence of antibody
0	5.0×10^3	3.5×10^2
1	4.5×10^4	4.0×10^2
10	6.0×10^4	6.5×10^2
100	9.0×10^4	7.0×10^2
1000	1.0×10^5	6.0×10^2

[a]. U937 cells were incubated with variable concentrations of rIFN-γ for 24 hours and infected with dengue virus at a m.o.i. of 5 p.f.u./cell in the presence of anti-dengue mouse serum at final dilution of 10^{-4}, or in the absence of antiserum. Cells were cultured at 3×10^5/ml in RPMI containing 10% FCS for 24 hours. Dengue virus contained in the culture fluids was titrated using a plaque titration assay.

C-2. IFN-γ produced by dengue-immune lymphocytes after stimulation with dengue antigen augments dengue virus infection of U937 cells

IFN-γ produced by human lymphocytes in vitro was used in the following experiments. PBMC from a dengue antibody-positive donor were cultured with dengue antigen for 7 days, and the culture fluid collected. The culture fluid contained 650 U/ml of IFN-γ and no detectable IFN-γ as determined by radioimmunoassays specific for human IFNγ and α. U937 cells were incubated for 24 hours with various dilutions of this culture fluid and infected with dengue virus-antibody complexes. The diluted culture fluids from lymphocytes of the dengue-immune donor that had been stimulated with dengue antigens and contained 10 or 100 U/ml of IFN-γ, augmented dengue virus infection of U937 cells as well as recombinant IFN-γ (Table 11).

Table 11: Culture fluid of a dengue-immune donor's PBMC stimulated with dengue antigen augments dengue virus infection of U937 cells

Source of IFNγ	Titer (U/ml)	Percentage of dengue antigen-positive cells[b]
None	0	11.5
Dengue culture fluid[a]	1	13.2*
	10	21.6**
	100	32.6***
Recombinant IFN-γ	100	42.6***

a The PBMC from a dengue-antibody positive donor were cultured with dengue antigen for 7 days, and culture fluid was collected. This culture fluid, which contained IFNγ at a titer of 650 U/ml and no detectable IFN-α as determined by RIA, was diluted to contain 1, 10, and 100 U/ml of IFNγ for pre-treatment of U937 cells.

b U937 cells were incubated with rIFN-γ or with dengue stimulated culture fluid for 24 hours, and were infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescence 24 hours after infection. The percentage of antigen positive cells was compared between IFN-γ pretreated cells and nontreated cells.

* $p>0.05$ (not significant)

** $p<0.01$

*** $p<0.001$

C-3. Anti-IFN-γ antibody inhibits IFN-γ-induced augmentation of dengue virus infections

To confirm that the IFN-γ contained in the culture fluid is responsible for the augmented dengue virus infection shown in Table 11, culture fluid which contained 10 U/ml of IFN-γ was incubated with a monoclonal anti-IFN-γ antibody, and then was used to treat U937 cells. Culture fluid pretreated with an anti-IFN-γ antibody did not augment dengue virus infection but the culture fluid pretreated an anti-IFN-γ antibody did augment (Table 12). We also tried to

block the effect of rIFN-γ using a monoclonal anti-IFN-γ antibody to confirm that the rIFN-γ-induced augmentation of infection was due to IFN-γ, and was not due to other substances derived from the production of this recombinant IFN-γ in E. Coli (3). U937 cells were pretreated with 10 U/ml of rIFN-γ which had been incubated with a monoclonal IFN-γ antibody or a polyclonal anti-IFNα antibody. An anti-IFN-γ antibody inhibited the augmenting effect of rIFN-γ, but anti-IFNα antibody had no effect (data not presented). These results confirm that IFN-γ is responsible for augmentation of dengue virus infection shown in Table 11 and Figures 3 and 4.

Table 12: IFN- contained in the culture fluid of PBMC is responsible for augmenting dengue virus infection of U937 cells

Source of IFN-γ	Antibodiess[c]	Percentage of dengue antigen-positive cells[d]
Dengue culture fluid[a]	None	66.0
	Anti-IFN-γ	20.5
	Anti-IFN-α	59.6
Control culture fluid[b]	None	20.9
None	None	15.0
	Anti-IFN-γ	16.4
	Anti-IFN-α	12.1

a Dengue culture fluid was obtained as described in the footnote of Table II, and was diluted to contain 10 U/ml of IFN-γ.

b PBMC of the same donor were cultured with a control antigen for 7 days, and a culture fluid was collected. This culture fluid, which contained no detectable IFN-γ or IFN-α was diluted similarly.

c Dengue culture fluid which contains IFN-γ was diluted to 10 U/ml and was then incubated with 1000 U/ml of monoclonal anti-IFN-γ and 2000 U/ml of anti-IFN-α at 4°C for 2 hours.

d U937 cells were incubated with culture fluids for 24 hours, and infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescent staining 24 hours after infection.

C-4. Human gamma globulin blocks IFN-γ-induced augmentation of dengue virus infection

It has been reported that IFN-γ increases Fcγ receptors on U937 cells. We tried to determine whether the IFN-γ-induced augmentation of dengue virus infection of Fcγ receptor medicated. U937 cells which had been treated with 100 U/ml rIFN-γ for 24 hours were incubated with γ-globulin at 4°C for 20 minutes. Cells were then infected with dengue virus-antibody complexes. γ-globulin inhibited infection by dengue virus antibody complexes of U937 cells which were pretreated with IFN-γ, whereas bovine serum albumin at the same concentration had no effect (Table 13). These results suggest that the IFN-γ induced augmentation of dengue virus infection is mediated by Fcγ receptors.

Table 13: Inhibition of IFN-γ induced augmentation of dengue virus infection by human γ-globulin

Blocking Reagent	Percentage of dengue Ag-positive cells[a]	
	IFN-γ pretreatment 100 U/ml	No IFNγ pretreatment
None	40.8	11.5
γ-Globulin (10 mg/ml)	1.8	<1.0
BSA (10 mg/ml)	41.6	11.6

a U937 cells which had been treated with 100 U/ml of rIFN-γ for 24 hours were incubated with human γ-globulin or bovine serum albumin at 4°C for 20 minutes. Cells were infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescence 24 hours after infection.

C-5. IFN-γ does not augment dengue virus infection of U937 cells in the presence of $F(ab')_2$ fraction of anti-dengue IgG antibody

We then used the $F(ab')_2$ fraction of anti-dengue IgG, to confirm that IFN-γ -induced augmentation of dengue virus infection is Fcγ receptor-mediated. Pretreatment of U937 cells with IFN-γ did not augment infection when cells were infected with dengue virus in the presence of $F(ab')_2$ prepared from anti-dengue IgG, but IFN-γ-pretreatment augmented infection when cells were infected with virus in the presence of purified anti-dengue IgG at 0.1 to 10 μg/ml (Table 14). This result confirms that the IFN-γ-induced augmentation of dengue virus infection is mediated by Fcγ receptors on U937 cells.

Table 14: $F(ab')_2$ prepared from anti-dengue IgG does not augment dengue virus infection of U937 cells pretreated with IFN-γ[a]

Final concentration of IgG and $F(ab')_2$ (μg/ml)	Percentage of dengue antigen-positive cells			
	IFN-γ pretreatment		No treatment	
	IgG	$F(ab')_2$	IgG	$F(ab')_2$
None	1.0	1.0	1.4	1.4
0.001	1.3	1.3	1.0	0.5
0.01	6.2	0.9	3.5	1.5
0.1	36.5*	2.1	9.8*	1.2
1	73.7*	0.4	34.2*	1.2
10	36.5*	<0.3	16.8*	<0.3
100	11.6**	0.4	7.0**	<0.3

a U937 cells were incubated with or without 100 U/ml of rIFN-γ for 24 hours, and infected with dengue virus at m.o.i. of 5 p.f.u./ml in the presence of various concentrations of purified anti-dengue IgG or $F(ab')_2$ prepared from IgG. The percentage of dengue antigen-positive cells was determined 24 hours after infection. The percentage of antigen positive cells was compared between IFN-γ-pretreated cells and nontreated cells at same concentrations of IgG and $F(ab')_2$.

* $p<0.001$;

** $p<0.005$; $p>0.05$ (not significant) at other concentrations of IgG and all the concentrations of $F(ab')_2$.

C-6. Augmentation of dengue virus infection correlates with increase in the number of Fcγ receptors

We tried to determine whether there is a correlation between the number of Fcγ receptors and the percentage of dengue antigen-positive cells. U937 cells were incubated with variable concentrations of IFN-γ for 24 hours, and examined for Fcγ receptor expression by quantitative FACS-analysis after exposure to MAb 32 which is specific for human FcγR1. The percentage of antigen positive cells was determined 24 hours after infection. The results shown in Figure 5 demonstrate that there is a good correlation between the percentage of dengue antigen-positive cells and the number of Fcγ receptors.

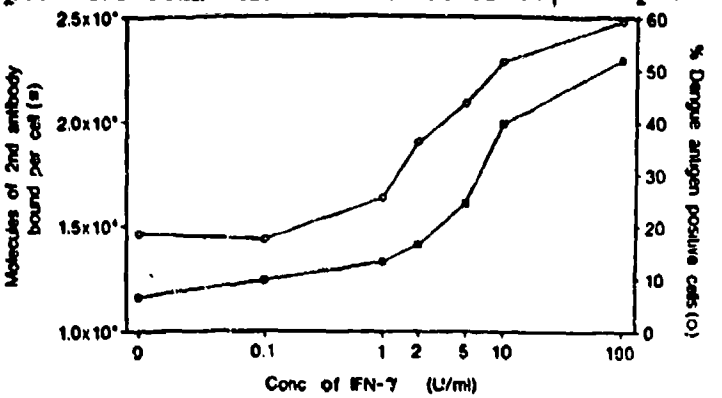

Figure 5: Correlation between IFN-γ increased Fcγreceptors and dengue virus infection. U937 cells were incubated with variable titers of IFN-γ for 24 hours. Cells were infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescent staining 24 hours after infection. The relative numbers of Fcγ receptors was measured by quantititative FACS-analysis.

C-7. Augmentation of dengue virus infection of human monocytes by IFNγ produced by PBMC in response to dengue antigen

We tried to determine whether the culture fluids of PBMC stimulated with dengue antigen could augment dengue virus infection of human monocytes. Culture fluids were obtained from dengue-immune PBMC after stimulation with dengue antigen, and they were then diluted to contain 3 U/ml of IFN. Culture fluids of the same dengue-immune PBMC that had been exposed to control antigen were similarly diluted. Human monocytes were exposed to these culture fluids for 24 hours. They were then washed and infected with dengue virus-antibody complexes. A similar percent of the monocytes cultured without addition of culture fluids from PBMC and those that were treated with the culture fluid from dengue-immune PBMC that had been exposed to control antigen became infected and expressed dengue antigens; however, an increased number of the monocytes treated with diluted culture fluids from dengue-immune PBMC that had been exposed to dengue antigen and contained 3 U/ml of IFNγ had dengue viral antigens (Table 15, $p<0.001$ in Exp. 1 and $p<0.01$ in Exp. 2). This augmenting effect of dengue antigen-stimulated culture fluid was abrogated by anti-IFNγ antibody, but not by anti-IFNα antibody (data not presented).

Table 15: Augmentation of dengue virus infection of human monocytes by IFNγ produced by PBMC in response to dengue antigen

Treatment of monocytes[a]	% dengue antigen-positive cells	
	Exp. 1	Exp. 2
None (RPMI containing 10% FCS)	22% (64/286)	5% (15/274)
Diluted culture fluid of PBMC stimulated with dengue antigen	37% (99/265)*	12% (38/325)***
Diluted culture fluid of PBMC stimulation with control antigen	24% (75/310**	5% (14/301)****

[a]Culture fluids obtained from dengue-immune PBMC after stimulation with dengue antigen were diluted to contain 3 U/ml of IFNγ, and culture fluids of the same PBMC exposed to control antigen, which did not contain detectable levels of IFNγ , were diluted similarly. After treatment with these fluids for 24 hours, human monocytes were infected with dengue virus type 2 at a m.o.i. of 10 p.f.u./cell in the presence of anti-dengue 2 mouse serum diluted at 1:10000. The percentage of monocytes infected with dengue virus was determined 24 hours after infection by indirect immunofluorescence.

The percentage of dengue anti-positive monocytes pretreated with culture fluids of PBMC stimulated with dengue antigen or control antigen were compared with the percentage of dengue antigen-positive monocytes which was not treated with culture fluid by Chi-square test. *$p<0.001$; **$p>0.5$ (not significant); ***$p<0.01$; ****$p>0.7$ (not significant).

III. DISCUSSION

The role of T lymphocytes in recovery from dengue virus infections and in the pathogenesis of severe complications has not been elucidated. In this report we demonstrated dengue-specific T lymphocyte proliferative responses with PBMC of dengue-antibody positive humans. We used fixed, sonicated dengue virus-infected Vero cells as antigens, as previously reported with measles virus (4), because cell-free preparations of live dengue virus did not stimulate PBMC of dengue-immune donors (data not presented). Proliferative responses induced by these antigens are dengue-specific, because PBMC of dengue antibody-positive donors respond to dengue antigens but not to control antigen, and PBMC of antibody-negative donors do not respond to dengue or control antigens. Antigens of the four dengue serotypes induced generally similar levels of proliferative responses of PBMC from the Thai adult donors. The dengue infection histories of these adult Thai donors are not known, but they all have high levels of antibodies to the four dengue serotypes, presumably as a result of prior natural infections. The PBMC of an American donor A, who had been immunized with yellow fever vaccine and later became infected with dengue 3 virus, responded best to dengue 3 antigen but also responded to dengue 1, 2 and 4 antigens to some degree (Table 3). These results suggest that human T cell responses to primary dengue infections include both type-specific and serotype cross-reactive responses.

We then established 15 dengue-specific T cell clones using donor A PBL to further analyze human T cell responses to dengue viruses. All the clones responded to dengue 3 Ag best, and they also responded to dengue 1, 2 and 4

antigens to lower but significant levels. These results suggest that T cell responses to dengue viruses are serotype cross reactive to varying degrees depending on the clone at the clonal level. We are defining the dengue epitopes which are recognized by human T lymphocytes. Dengue virus has three structural proteins; envelope protein (E), membrane protein (M) and capsid protein (C) (5,6). It also induces seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (5,7). Although the role and immunogenic properties of these ten proteins has not been defined, E protein is thought to contain epitopes which induce neutralizing antibody responses (8). Monoclonal antibodies to E protein protect mice from lethal dengue infection (9). Recently, it has been reported that immunization of mice with dengue 2 virus NS1 protein provided significant protection against lethal challenge with dengue 2 virus (10). Identification of the epitopes or dengue antigens which are recognized by T cells and may have a role in protection or in the pathogenesis of DHF and DSS is an important area for research.

In this report we also demonstrated that dengue-specific $CD4^+$ T cell clones produce IFNγ after stimulation with dengue antigens. We examined the effect of IFNγ on dengue virus infection of human monocytic cells, and demonstrated that IFN-γ augments dengue virus infection of U937 cells in the presence of anti-dengue antibodies. This effect is Fcγ receptor-mediated, because (i) IFN-γ has no augmenting effect on the infection of U937 cells when cells were infected with dengue virus in the absence of anti-dengue antibody, (ii) IFN-γ does not augment dengue virus infection when cells were infected with virus complexed to the $F(ab')_2$ fraction prepared from anti-dengue IgG, (iii) IFN-γ has no augmenting effect when Fcγ receptors on U937 cells are blocked by γ-globulin, and (iv) there is a good correlation between the percentage of dengue antigen-positive cells and the number of Fcγ receptors on U937 cells. We observed that IFNγ increases the number of Fcγ receptors on U937 cells as previously reported by other investigators (11,12). Based on these observations, we conclude that the increase in the number of Fcγ receptors on U937 cells induced by IFN-γ leads to an augmented uptake of dengue virus in the form of dengue virus-antibody complexes, which results in a higher percentage of dengue infected cells, and in higher yields of infectious dengue virus. IFNγ also augmented dengue virus infection of human monocytes enriched from peripheral blood mononuclear cells, when monocytes were infected with virus in the presence of anti-dengue antibodies. IFNγ did not augment dengue virus infection of human monocytes when cells were infected in the absence of antibody (data not presented).

It has been reported that IFN-α has no or little effect on the number of Fcγ receptors on monocytic cells (11,12). We found that IFN-α suppresses dengue virus infection of U937 cells at doses higher than 1 U/ml even when cells are infected in the presence of anti-dengue antibody (data not presented). We attribute this suppressed effect to the antiviral activity of IFN-α. We recently reported that high levels of IFNα were produced by dengue virus-infected monocytes (13) and by DR+, non-T lymphocytes cultured with dengue-infected monocytes (14), furthermore the IFNα produced was active in limiting infection of human monocytes by dengue virus (13,14).

It has been hypothesized that increased infection of monocytes with dengue virus in the form of dengue virus-antibody complexes may occur in vivo and play

an important role in the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2). This is supported by epidemiological studies which reported that most cases of DHF/DSS occur during secondary dengue infections when anti-dengue antibodies are present (15,16). We have also found that the T lymphocytes of individuals who have antibodies to dengue viruses proliferate and produce high titers of IFN-γ after stimulation with dengue antigens in vitro. The IFN-γ produced by dengue virus stimulation of immune T cells was active in augmenting dengue virus infection of U937 cells and human monocytes. Therefore, we hypothesize that IFN-γ is produced by dengue-specific T lymphocytes during secondary dengue infections after stimulation with conserved dengue antigens and the IFN-γ produced might contribute to the pathogenesis of DHF/DSS by enhancing Fcγ receptor expression on human monocytes, thereby increasing the number of infected monocytes and the yields of infectious dengue virus in the presence of anti dengue virus-antibodies.

IV. REFERENCES

1. Halstead, S.B. 1980. Immunological parameters of togavirus disease syndromes. In The Togaviruses: Biology, Structure, Replication. R.W. Schlesinger, editor. Academic Press, New York. 107-173.

2. Halstead, S.B. 1981. The pathogenesis of dengue: molecular epidemiology in infectious disease. Am. J. Epidemiol. 114:632-648.

3. Caray, P.W., D.W. Geungi, D. Pennica, R. Yelverton, R. Narajan, C.C. Simonsen, R. Derynck, P.J. Sherwood, D.M. Wallace, S.L. Berger, A.D. Levinson, and D.V. Goeddel. 1982. Expression of human immune interferon cDNA in E. Coli and monkey cells. Nature (Lond) 295:503-508.

4. Richert, J.R., H.F. McFarland, D.E. McFarlin, A.H. Johnson, J.N. Woody, and R.J. Hartzman. 1985. Measles-specific T cell clones from a twin with multiple sclerosis: genetic restriction studies. J Immunol. 134:1561-1566.

5. Westaway, E.G. 1987. Flavivirus replication strategy. Adv. Virus Res. 33:45-90.

6. Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R.M. Chanock, C.J. Lai, and Y. Makino. 1986. Cloning full-length dengue 4 type 4 viral DNA sequences: Analysis of genes coding for structural proteins. Virology 155:77-88.

7. Mackow, E., Y. Makino, B. Zhao, Y.M. Zhang, L. Markoff, A. Buckler-White, M. Guiler, R. Chanock, and C.J. Lai. 1987. The nucleotide sequence of dengue type 4 virus: Analysis of genes coding for nonstructural proteins. Virology 159:217-228.

8. Russell, P.K., W.E. Brandt, and J.M. Dalrymple. 1980. Chemical and antigenic structure of flaviviruses. In The Togaviruses: Biology, Structure, Replication. R.W. Schlesinger, editor. Academic Press, New York. 503-529.

9. Kaufman, B.M., P.L. Summers, D.R. Dubois, and K.C. Eckles. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hug. 36:427-434.

10. Schlesinger, J.J., M.W. Brandriss, and E.E. Walsh. 1987. Protection of mice against dengue 2 virus encephalitis by immunization with dengue 2 virus non-structural glucoprotein NS1. J. Gen. Virol. 68:853-857.

11. Guyre, P.M., P. Morganelli, and R. Miller. 1983. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. J. Clin. Invest. 72:393-397.

12. Perussia, B., E.T. Dayton, R. Lazarus, V. Fanning and G. Trinchieri. 1983. Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. J. Exp. Med. 158:1092-1113.

13. Kurane, I., and F.A. Ennis. 1988. Production of interferon alpha by dengue virus-infected human monocytes. J. Gen. Virol. 69:445-449.

14. Kurane, I., and F.A. Ennis. 1987. Induction of interferon-α from human lymphocytes by autologous, dengue virus-infected monocytes. J. Exp. Med. 166:999-1010.

15. Burke, D.S., A. Nisalak, D.E. Johnson, and R.M. Scott. 1988. A prospective study of dengue infections in Bangkok. Am. J. Trop. Med. Hyg. 38:172-180.

16. Sangkawibha, N., S. Rojanasuphot, S. Ahandrik, S. Viriyapongse, J. Jatanasen, V. Salitul, B. Phanthumachinda, and S.B. Halstead. 1984. Risk factors in dengue shock syndrome: A prospective epidemiologic study in Rayong, Thailand. Am. J. Epidemiol. 120:653-669.

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